CACNA1C methylation: association with cortisol, perceived stress, rs1006737 and childhood trauma in males

PENNINGTON, Kyla, KLAUS, Kristel, FACHIM, Helene A., BUTLER, Kevin, TRISCHEL, Ksenia, DALTON, Caroline <http://orcid.org/0000-0002-1404-873X>, HEALD, Adrian and REYNOLDS, Gavin P.

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Abstract (120 words max)

Aim: We investigated morning cortisol, stress, rs1006737 and childhood trauma relationship with *CACNA1C* methylation. **Materials & Methods:** Morning cortisol release, childhood trauma and perceived stress were collected and genotyping for rs1006737 conducted in 103 adult males. Genomic DNA extracted from saliva was bisulphite converted and using pyrosequencing methylation determined at 11 CpG sites within intron 3 of *CACNA1C*. **Results:** A significant negative correlation between waking cortisol and overall mean methylation was found and a positive correlation between CpG5 methylation and perceived stress. **Conclusion:** *CACNA1C* methylation levels may be related to cortisol release and stress perception. Future work should evaluate the influence of altered *CACNA1C* methylation on stress reactivity to investigate this as a potential mechanism for mental health vulnerability.

Summary points (8-10 bullet point sentences highlighting key points of the article)

- *CACNA1C*, encoding the Cav1.2 subunit of the L-type calcium channel, is a cross-disorder risk gene implicated in many psychiatric disorders.
- It has been previously shown that alterations in CACNA1C expression may influence the stress response system through impacting on glucocorticoid receptor activity.
- Previous work in our lab has shown that healthy adult males carriers of the minor allele of rs1006737 in *CACNA1C* who have experienced childhood trauma have a lower cortisol awakening response.
- DNA methylation, an epigenetic factor known to regulate gene expression, might be one mechanism through which alterations in *CACNA1C* affects the stress response system.
- No significant relationship was found between rs1006737 and childhood trauma on *CACNA1C* methylation levels.
- Increasing waking cortisol levels were significantly correlated with decreased overall mean methylation of *CACNA1C* in adult males
- Altered methylation levels of *CACNA1C* may be related to measures of stress perception and HPA axis function in healthy adult males.

Keywords

CACNA1C; rs1006737; DNA methylation; childhood trauma; cortisol; stress.

Introduction

One of the most robust and replicated findings in psychiatric genetics research is that many genes are likely to contribute small but significantly increased risk to multiple disorders [1, 2]. An example of a

well replicated cross-disorder risk gene is *CACNA1C*, implicated primarily in bipolar disorder but also in schizophrenia, attention deficit hyperactivity disorder, major depression, autism and post-traumatic stress disorder [3-7].

CACNA1C encodes the alpha (1C) subunit of the Ca(v)1.2 voltage-gated L-type calcium channel (LTCC) predominantly expressed in brain (80%) but also in heart and endocrine cells [8]. In the brain, Ca(V)1.2 are involved in many key processes including neuronal signalling and neuronal plasticity [9]. In addition, L-type calcium channels are known to be highly responsive to glucocorticoids [10, 11] with administration of cortisol leading to increased expression in the brain [12, 13]. Endophenotypes such as altered hypothalamic pituitary adrenal (HPA)-axis function and impaired cognitive processes likely contribute to the onset of mental health disorders hence elucidating the mechanisms by which risk genes might contribute towards these known risk factors are increasingly valid and important targets for research [14].

Enhancement of voltage dependant calcium conductance in response to glucocorticoid activation, a key component of HPA axis function and stress reactivity, has been known about for some time [11] with more recent work showing that chronic stress is also directly linked to elevated calcium current amplitude and increased Cav1.2 mRNA expression [12, 13, 15]. This elevation may be necessary for mediation of the stress response and as part of the positive feedback effect of cortisol on the brain with elimination of *CACNA1C* from the forebrain in mice resulting in increased anxiety [16] and depletion during embryonic development leading to increases in susceptibility to chronic stress [17]. Interestingly, deletion in adulthood had the opposite effect resulting in increasing cognitive flexibility, strengthening synaptic plasticity and inducing stress resilience in mice [17] suggesting that timing is crucial to the effect of altered *CACNA1C*. A neuroimaging and post-mortem study [18] found increased hippocampal activation during emotional processing and increased prefrontal activity, often interpreted as prefrontal inefficiency, during executive cognition in the risk associated allele homozygotes of the *CACNA1C* single nucleotide polymorphism (SNP) rs1006737 (A/G). This study also reported increased brain levels of CACNA1C mRNA in A-carriers although it should be noted that expression levels have been shown to differ depending on brain region [19]. Functional characterisation of several key SNPs within the sequence encoding for *CACNA1C* has revealed 16 SNPs in high linkage disequilibrium with rs1006737 (Eckart et al., 2016). This region, including the 16 SNPs, was also shown to interact with the *CACNA1C* promoter and other potential regulatory regions [19] with the authors concluding that rs1006737 may be a quantitative trait locus for *CACNA1C* transcript levels.

There has been substantial interest in researching the effects of rs1006737 gene variant. A-carriers of rs1006737 have been found to have lower activation than non-risk (GG) homozygotes in the right

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hippocampus during an episodic memory task in a sample of 63 healthy males [20]. In a subsequent study conducted with 540 healthy males, A-allele carriers of the same SNP were found to have significantly reduced extraversion, increased harm avoidance, increased trait anxiety, increased paranoid ideation and higher startle reactivity [21]. We recently showed that the rs1006737 polymorphism of *CACNA1C* may partially moderate the effects of early life stress on HPA-axis function as measured by morning rise in cortisol (0-30 minutes after waking) in a sample of healthy adult males [22]. More specifically, GG allele homozygotes but not A allele carriers, who had experienced trauma, were found to have a significantly heightened rise in morning salivary cortisol levels in adulthood in comparison to those who had not experienced trauma. This may be a reflection of adaptation to stress exposure during childhood. The current study aimed to extend these findings to investigate the relationships between DNA methylation of *CACNA1C*, waking cortisol and perceived stress in healthy male adults, and also whether there was an interaction effect of *CACNA1C* rs1006737 genotype and childhood trauma on *CACNA1C* methylation levels. Given the evidence for the important role of Cav1.2 in the glucocorticoid response in addition to the evidence that genetic variation in *CACNA1C* can confer risk to the development of mental health disorders, we hypothesised that *CACNA1C* methylation levels would be significantly related to current perceived stress and cortisol release. We also hypothesised that there would be an interactive effect of childhood trauma and genotype on methylation levels with those individuals who had experienced childhood trauma and were carriers of the risk allele having higher methylation levels than those who had not experienced childhood trauma.

Materials & Methods

Participants

Genomic DNA was extracted from saliva donated by a subset of 103 males, recruited as part of a previously published local community based UK sample [22, 23]. Participants were of Caucasian ethnicity, and none of the participants had a current diagnosis of a psychiatric disorder, drug or alcohol addiction problems, nor used steroid based medication at the time of recruitment as determined through self-report. Individuals were genotyped for rs1006737 variant (A/G) within intron 3 (position 80 2236129) of the *CACNA1C* gene (GRCh37.p13 or hg19 (GCF_000001405.25); Chr:12, NC_000012.11 (2079952..2807115), also reported previously [22]. In order to evaluate the potential influence of rs1006737 variation on transcription, transcription factor (TF) binding sites were identified using the tool Promo Alggen http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3. All processes and recruitment was conducted following institutional ethical review board approval and informed consent obtained for all participants involved.

Early life stress, perceived stress, waking cortisol and cortisol awakening response

Childhood trauma scores in this sample were calculated based on responses to the Childhood Traumatic Events Scale, CTES [24]. Potential symptoms of current psychopathology were assessed with Hospital Anxiety and Depression Scale, HADS [25]. Current perceived stress levels were measured using the Perceived Stress Scale, PSS-14 [26]. Cortisol awakening response (CAR), was calculated from a mean morning cortisol release in saliva (30 minutes after waking – waking cortisol levels) over two consecutive days. Associated factors such as waking time and hours of sleep were recorded [23]. All study procedures were approved by the School of Psychology Research Ethics Committee (SOPREC) at the University of Lincoln.

Methylation analysis

Genomic DNA was bisulphite-modified to convert unmethylated cytosine residues to uracil using the EpiTec Fast DNA Bisulphite Kit (Qiagen) with a calculated mean conversion of 99%. A pyrosequencing method was developed to analyse 11 CpG sites of the *CACNA1C* gene (Figure 1), previously investigated in the context of suicide attempters [27] and the sequence was amplified by PCR using primers, including a biotinylated reverse primer. PROMO-ALGGEN tool was used to identify transcription factors binding sites nearby the CpG island and SNP variant rs1006737 (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). We performed the experiments in duplicates and the results are shown as mean value. The samples were randomized across the plates and experiments conducted blind to avoid introducing bias

Figure 1 about here

Figure 1. Schematic of calcium voltage-gated channel subunit alpha1 C human gene (*CACNA1C*: Chromosome 12, NC_000012.12 (1969552..2697950), 12p13.33 showing the CpGs chosen to be analysed in intron 3 (in bold, numbered 1 to 11 and underlined), the SNP (rs1006737) position in red and blue boxes representing the binding sites for GRα. Adapted from (Kantojarvi et al., 2017).

PCR reactions were carried out with 20 ng bisulphite-converted DNA using the PyroMark PCR kit in a final volume of 25 µl containing 12.5 µl 1x PyroMark PCR Master Mix, 2.5 µl 1x CoralLoad Concentrate, 1 µl of each primer in a final concentration of 0.05 µM, 8 µl RNase-free water. Amplification conditions were done as follows: 95°C for 15 min, 45 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 30 s, finally, 72°C for 10 min. Methylation status in the sequence of *CACNA1C* was determined with a PyroMark Q48 pyrosequencer (Qiagen UK) using 10 µl PCR product and a sequencing primer. Pyrosequence setup and data reading were conducted by PyroMark Q48 2.4.2 software. Samples were submitted to PCR and pyrosequencing in duplicate; any inconsistencies between samples were resolved following further repetition. All the primers used for the amplification and sequencing are listed in the Table 1.

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Statistical analysis

All statistical analyses were conducted in SPSS version 23 (IBM, 2015). All CpG sites methylation levels were significantly correlated consequently it was considered meaningful to combine methylation levels to form an overall mean methylation level across all sites. Age was also found to correlate with overall mean methylation levels and methylation levels in 7 of the individual 11CpG sites investigated and consequently was included as a covariate in all analyses conducted. To investigate any difference in methylation levels between individuals in the two genotype groups, two way ANCOVAs with *CACNA1C* genotype (AA/AG or GG) and childhood trauma (yes/no) as main factors and age as a covariate were conducted on mean methylation levels then each CpG site separately. Subsequently, rise in morning cortisol, waking cortisol and current perceived stress were analysed using partial Spearman correlational analysis adjusting for age to investigate relationships with overall mean methylation levels and then each CpG site separately. For the individual CpG site analyses corrections were made for multiple comparison using Bonferroni ($p<0.005$), with mean methylation analyses considered significant at $p<0.05$.

Results

Demographics, rs1006737 and CAR

Methylation was not successful for two samples and there were single missing data on methylation levels at various CpG sites with no more than four samples failing for each CpG site except for CpG6 which did not reach the threshold for 10% missing data and was subsequently removed from all analysis. Consequently subsequent statistical analyses were conducted with 101 subjects for the ANCOVAs and with 97-101 participants for the partial correlation analysis. There were 56 individuals who were AA/AG and 45 who were identified as GG homozygotes for rs1006737. In addition, 38 were found to have been exposed and 63 not exposed to childhood trauma. Across the genotype groups there were found to be no significant differences between the *CACNA1C* gene rs1006737 AA/AG and GG groups in terms of age, years of education, sleep duration, waking time, current perceived stress (PSS-14), anxiety and depression (HADS), reported childhood trauma experiences (and CTES), waking cortisol, and mean CAR ($p>.05$ in all cases, Table 2). The A-allele carriers had a blunted cortisol response at 30 min post awakening in comparison with the non-carriers ($F=4.126$, $p=.045$). Additionally, through using the tool Promo Alggen we found that the presence of the the G allele extinguishes the binding site for glucocorticoid receptor β (GRβ) which is present in the presence of the A allele.

Table 2 about here

Methylation levels

2x2 ANCOVA analysis with genotype and childhood trauma as main factors and methylation levels at CpG sites 1-11 and overall mean methylation levels as dependent variables revealed no significant interaction effect of genotype and childhood trauma on *CACNA1C* methylation levels, p>.05 in all cases. There was no main effect observed between any of the individual CpG methylation sites and genotype after correction for multiple testing (see Figure 2a). There was no main effect observed between childhood trauma and *CACNA1C* methylation levels (See Figure 2b).

Figure 2 about here

Correlational analysis revealed a significant negative association between overall mean methylation levels (without CpG6) and waking cortisol and a weakly positive association with PSS-14 (p=0.065). There was no relationship with mean methylation and morning rise in cortisol. When looking at individual CpG site methylation levels a negative association between CpG2, 9 and 10 methylation and waking cortisol were found to be nominally significant. There was an also significant positive association between current perceived stress (PSS-14) and *CACNA1C* methylation reaching nominal significance at CpG5. There were no associations between cortisol awakening response and *CACNA1C* methylation levels, *p*>.05 in all cases. Bonferroni correction resulted in a lack of significant association with any individual CpG site. See Table 3 for all correlational analyses.

Table 3 about here

Discussion

This study aimed to investigate the relationship between factors related to stress (waking cortisol, morning cortisol release and current perceived stress) on *CACNA1C* methylation levels in 11 CpG sites in intron 3 in a cohort of healthy adult males. Our study also looked at whether a genetic variation in *CACNA1C* (rs1006737), implicated in the control of expression of this gene, along with the experience of childhood trauma, was associated with differences in *CACNA1C* methylation levels. We found that increasing mean methylation of *CACNA1C* was significantly correlated with lower levels of waking cortisol which was reflected in negative correlations at all CpG sites individually . Consistent weakly positive correlations of methylation at each CpG site (CpG5 in particular) were found with perceived stress and in overall mean methylation levels, though these were not statistically significant. In addition, we found no evidence of any interactive effect of childhood trauma and rs1006737 genotype on *CACNA1C* methylation levels after correction for multiple testing. Thus, this preliminary data suggests

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there may be a role for *CACNA1C* methylation in regulating and responding to glucocorticoid activity extending previous work indicating that increased levels of glucocorticoids results in increased levels of CACNA1C mRNA and intra-cellular calcium [28].

We identified a significant relationship between mean methylation of *CACNA1C* CpG sites and waking cortisol in which higher methylation was associated with reduced cortisol, an effect most strongly observed at three out of the ten individual CpG sites analysed. Higher mean methylation levels overall of the CpG sites investigated within the *CACNA1C* gene in the current study were significantly related to lower waking free salivary cortisol levels and this directional relationship could be seen to a greater or lesser extent across all of the individual CpG sites when looked at individually. Although this does not extend our previous findings directly in terms of showing a potential mechanism for the interaction we observed between *CACNA1C* genotype and childhood trauma in influencing the cortisol awakening response [22] the findings of the current study show more generally support for a relationship with *CACNA1C* and measures of stress reactivity. Thus *CACNA1C* genotype and *CACNA1C* methylation are suggested to independently confer vulnerability to mental health risk and one of the mechanisms of this is suggested to be via alterations of stress responsivity via the HPA-axis. Given previous work showing the activation of L-type calcium channels in response to stress [28] it may be that the finding of the current study reflects reduced gene transcription and Ca*v*1.2 protein expression through increased methylation in response to lowered circulating cortisol levels. It has been previously proposed that methylation may mediate adaptive and maladaptive responses to stress, in some cases being protective and in others potentially increasing vulnerability [29]. Consequently, it may be that increased *CACNA1C* methylation levels and reduced activation of L-type calcium channels represent a reduced adaptive capacity for stress leading to increased cortisol levels/HPA axis activation. **Figure 3** speculatively indicates how these relationships may lead to a less adaptive stress response in some individuals. However our findings in relation to PSS-14 and *CACNA1C* methylation were relatively weak and do not survive correction for multiple comparisons. Further work is clearly needed to explore the causal mechanisms behind these findings and confirm the speculative interpretation presented here.

Figure 3 about here

It is important to mention that within the CpG island we chose to analyse there are many binding sites for transcription factors (TFs), and interestingly, multiple sites for the TF glucocorticoid receptor alpha (GRα) across different CpGs. It has been demonstrated that DNA methylation can regulate the transcriptional activity of the GRα via post-translational modifications of the receptor protein [31], in other words, DNA methylation can modify the binding sites of the GRα receptor, which allow us to suggest that increased mean methylation levels found in *CACNA1C* across all CpG sites could be modulating the activity of GRα. This increased methylation may relate to the association with lower waking free salivary cortisol levels found in our study. Since GRβ exerts a dominant negative effect

on GRα [30]and as higher levels of GRβ has been found to correlate with reduced activity of glucocorticoids, the extinguishing of the binding site for GRβ in the non-risk allele may be another regulatory mechanism which explains some of our findings. It is also of note that intronic methylation of *FKBP5*, another key cross disorder risk gene involved in the regulation of glucocorticoid receptor activity, has been shown to be affected by trauma and psychiatric intervention and that the mechanism for this may be due to the existence of glucocorticoid response elements thought to regulate expression of FKBP5 [31, 32].

It is well established that gene variants in introns can affect gene expression directly [33, 34] and that DNA methylation in introns has been shown to affect gene expression [35]. In addition, intron 3 of *CACNA1C* has been shown to be important in the regulation of *CACNA1C* gene expression potentially via interactions of this sequence with an enhancer loop [19]. The SNP investigated in this study is likely in linkage disequilibrium with multiple other SNPs in intron 3 suggesting that the regulatory region for this gene is large and includes rs1006737 and the CpG island investigated in this study. It has been proposed that there may also be multiple isoforms of Ca*v*1.2 due to alternative transcription start sites with the coding sequence starting from exon 4 or later [36] and that there are alternative isoforms of *CACNA1C* [37] implying that the transcription factors within intron 3 may also influence gene expression levels of certain isoforms.

Previous research has also shown that increased methylation may confer susceptibility to the development of mental health problems. Indeed, increasing methylation levels in some of the same CpG sites investigated in the current study have been shown to be related to increasing scores on the Barratt Impulsivity Scale (BIS-11) in a healthy control group and several also related to altered brain activation in suicide attempters [27]. This supports and extends the findings of the current study by suggesting that higher levels of methylation at the same CpG sites may be related to neural processes related to mental health disorders and subclinical risk factors such as increased impulsivity and thalamic expression of CACNA1C. This relates to the findings of the current study with respect to understanding mental health risk more generally in terms of transdiagnostic factors such as stress sensitivity. In healthy adults, it has also been shown that rs1006737 risk allele homozygotes (AA) have increased hippocampal and amygdala activity during emotional imaging tasks in comparison to non-risk allele carriers [18]. This study also investigated mRNA expression levels of *CACNA1C* showing risk allele homozygotes to have the highest expression levels. Bigos and colleagues did not report a relationship with age and mRNA expression levels, in contrast to the finding in this study of significantly increased methylation levels across 8/11 of the CpG sites with increasing age. Other studies have also investigated the effect of *CACNA1C* gene variants with structural and functional abnormalities in the brain in MDD, BPD and SCZ [38, 39]. In support of our findings related to age, perceived stress and methylations

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levels are findings that depletion of *CACNA1C* during embryonic development, but not adulthood, increases susceptibility to chronic stress in mice and that embryonic deletion of *CACNA1C* in forebrain glutamatergic neurons is linked to endophenotypes linked to psychiatric disorders such as increased anxiety, reduced sociability and impaired synaptic plasticity [17]. Altered gene expression levels have also been reported in both studies in the human cerebellum and induced neurons [40] in addition to work on induced neurons which showed significant functional alterations in L-type voltage gated calcium channel current density and mRNA expression of *CACNA1C* in risk homozygotes compared to non-risk carriers [41].

It should be mentioned that we did not find a relationship between the average rise in morning cortisol as sampled in this study over two days and at two time points (0 and 30 minutes after waking) and methylation status. There are several reasons why this might be but one of these might be due to the limitations of the CAR data collected from this sample. This may relate to the quality of the data whereby the sampling procedure could have been improved [42] and the variability of the data means that a larger sample may be needed in order to see an effect. In addition, in this sample, we did not find an interaction between genotype and childhood trauma on methylation levels nor a main effect of childhood trauma. Further limitations around this sample have been discussed previously [22, 23] but the fact that our sample was restricted to adult males is important in the context of the current study due to findings in rats of a sex-dependent effect of *CACNA1C* haploinsufficiency in affecting behavioural inhibition in response to a stressor (Wohr et al., 2019). Future work should also investigate the influence of *CACNA1C* methylation levels on stress responsiveness to acute stress in both a male and female sample and how this might potentially link to mental health vulnerability.

Future Perspective

This work shows for the first time that alterations in mean methylation levels of multiple CpG sites within intron 3 of *CACNA1C* may be related to waking cortisol, a measure of HPA axis function in healthy adult males. Our findings also tentatively suggest altered *CACNA1C* methylation levels might also be related to altered stress perception and we have put together a speculative figure which illustrates the relationships we have observed (**see Figure 3**). In our opinion the findings reported in the current study, in addition to previously published work investigating the impact of glucocorticoids on calcium channel activity provide evidence for an important role for calcium channels in altering stress thresholds, sensitivity and adaptiveness. Future work should explore *CACNA1C* methylation levels during development given previous work looking at post-mortem mRNA expression levels in the prefrontal cortex and the reported relationship with age in the current study. Rigorous and methodical analysis of methylation levels at different ages throughout childhood and adolescence will help elucidate the importance of *CACNA1C* methylation levels at key periods of brain development and in adulthood in relation to HPA axis function and potentially stress perception. In addition, given the

greater number of females diagnosed with affective disorders including anxiety and depression, it is expected that additional investigations are likely to reveal a greater effect in females than that observed in the male population in the current study. Large scale database and computer modelling in this area will also be fruitful areas to explore (e.g.[43]). Indeed, a multidisciplinary approach will be the most successful in clarifying further the effect of *CACNA1C* methylation and gene activation more directly on stress responsivity and perception leading to an improved understanding of mental health vulnerability more generally.

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Reference annotations:

(6-8 references of particular significance to the subject under discussion with 1-2 line synopsis)

*of interest

**of considerable interest

*Cross-Disorder Group of the Psychiatric Genomics C. Identification of risk loci with shared effects on five major psychiatric disorders: a genome-wide analysis. *Lancet* 381(9875), 1371 1379 (2013).

- **Genetic evidence for risk loci implicating *CACNA1C* across multiple psychiatric disorders.**

**Moon AL, Haan N, Wilkinson LS, Thomas KL, Hall J. CACNA1C: Association With Psychiatric Disorders, Behavior, and Neurogenesis. *Schizophr Bull* 44(5), 958-965 (2018).

- **Overview of findings regarding *CACNA1C* variation in animal models**.

*Bhat S, Dao DT, Terrillion CE *et al*. CACNA1C (Cav1.2) in the pathophysiology of psychiatric disease. *Prog Neurobiol* 99(1), 1-14 (2012).

- **Review of how *CACNA1C* might contribute to pathophysiology of psychiatric disorders.**

*Klaus K, Butler K, Gutierrez H, Durrant SJ, Pennington K. Interactive effects of early life stress and CACNA1C genotype on cortisol awakening response. *Biol Psychol* 136 22-28 (2018).

- **Earlier study reporting an interaction effect between CACNA1C genotype and childhood trauma on morning cortisol release.**

*Kim YJ, Park HJ, Jahng GH *et al*. A pilot study of differential brain activation to suicidal means and DNA methylation of CACNA1C gene in suicidal attempt patients. *Psychiatry Res* 255 42-48 (2017).

- **Evidence for potential role for CACNA1C methylation levels and brain activation patterns in suicide attempters.**

**Bali A, Gupta S, Singh N, Jaggi AS. Implicating the role of plasma membrane localized calcium

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channels and exchangers in stress-induced deleterious effects. *Eur J Pharmacol* 714(1-3), 229 238 (2013).

- **Review discussing mechanisms involved in stress-induced rise in intracellular calcium levels and implications.**

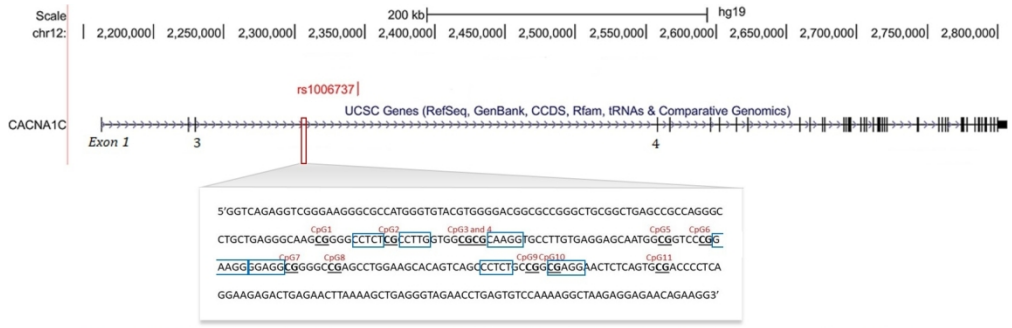

Figure 1. Schematic of calcium voltage-gated channel subunit alpha1 C human gene (*CACNA1C*: Chromosome 12, NC_000012.12 (1969552..2697950), 12p13.33 showing the CpGs chosen to be analysed in intron 3 (in bold, numbered 1 to 11 and underlined), the SNP (rs1006737) position in red and blue boxes representing the binding sites for GRα. Adapted from (Kantojarvi et al., 2017).

Figure 1. Schematic of calcium voltage-gated channel subunit alpha1 C human gene (CACNA1C: Chromosome 12, NC_000012.12 (1969552..2697950), 12p13.33 showing the CpGs chosen to be analysed in intron 3 (in bold, numbered 1 to 11 and underlined), the SNP (rs1006737) position in red and blue boxes representing the binding sites for GRα. Adapted from (Kantojarvi et al., 2017).

290x125mm (150 x 150 DPI)

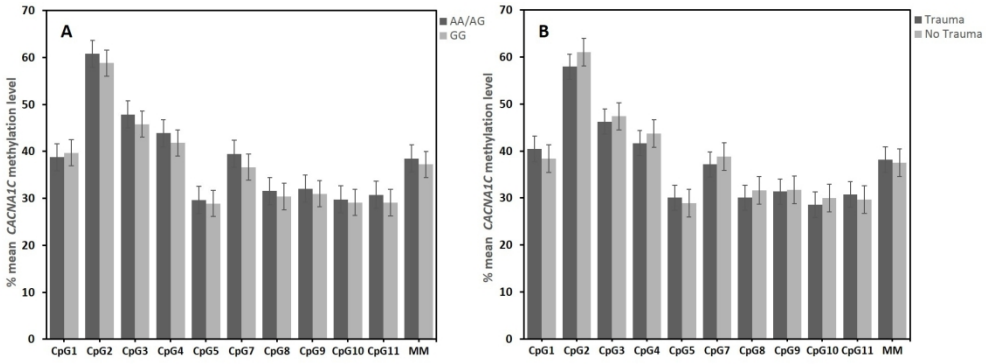

Figure 2. a) Mean percentage methylation at eleven CpG sites within *CACNA1C* risk allele carriers (AA/AG) (n=56) and non-carriers (GG) (n=45) and; **b)** Mean percentage methylation at ten CpG sites within *CACNA1C* gene in individuals with (n=38) and without (n=63) reported childhood trauma experience. The experiments were conducted in duplicates and the results are shown as mean value.

See attached figure.

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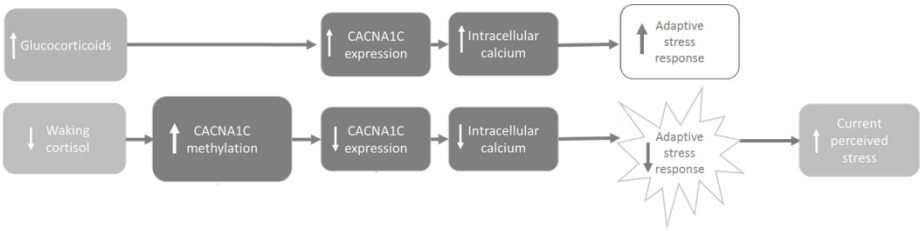

Figure 3 Theoretical model of potential pathway by which *CACNA1C* methylation might be involved in stress sensitivity. Previously published research has shown that calcium channel activity is essential for a healthy and adaptive stress response (indicated in top pathway). Findings of the current study suggest that *CACNA1C* methylation may be related to this via response to waking cortisol levels which may result in altered expression levels of CACNA1C and consequently reduced intracellular calcium. This is proposed to result in a less adaptive stress response system and altered threshold for perception of stress (lower pathway).

See attached for legend.

314x131mm (150 x 150 DPI)

	Primer sequences
CACNA1C	F 5' TTGAGTAGTTAGGGTTTGTTGAGG 3'
	R [BIO] 5' CCCTCAACTTTTAAATTCTCAATCTCTTC 3'
	Seq 1 5' GGGTTTGTTGAGGGTA 3'
	Seq 2 5' GTTTAGGAAGGGGAGG 3'

Table 1. List of Forward (F) and biotinylated Reverse (R) primers used in PCR reactions, and Sequencing (Seq) primers for pyrosequencing.

177x71mm (150 x 150 DPI)

Characteristics	AA/AG (n=56)	GG (n=45)	p-value
Age	35.23 (10.60)	33.73 (11.08)	.490
Years of education	14.16 (2.44)	14.62 (2.24)	.330
HADS Anxiety	6.57 (3.72)	6.31 (3.78)	.729
HADS Depression	3.07 (2.43)	3.33 (2.80)	.617
PSS-14 Perceived Stress	20.43 (8.83)	20.91 (7.59)	.772
Reported CT (%)	37.5	37.7	.963
CTES Score	7.34 (6.59)	8.53 (6.52)	.366
Sleep duration (hours)	6.72 (1.37)	6.64 (1.19)	.766
Waking time	7:24 (1:04)	7:30 (1:07)	.651
Waking cortisol (nmol/L)	6.87 (3.96)	8.23 (4.66)	.115
+30 min cortisol (nmol/L)	8.22 (4.70)	10.32 (6.06)	.045*
Mean CAR (nmol/L)	1.35 (3.25)	2.16 (5.25)	.344

Notes: Data shown are means and standard deviations in brackets; HADS= Hospital Anxiety and Depression Scale (Zigmond & Snaith, 1983); PSS-14= Perceived Stress Scale (Cohen, Kamarck, & Mermelstein, 1983); CT= Childhood Trauma; CTES= Childhood Traumatic Events Scale (Pennebaker & Susman, 1988); CAR= Cortisol Awakening Response; *p<.05.

Table 2. Comparison of age, years of education, HADS and PSS-14 scores, percentage of reported childhood trauma, CTES scores, sleep duration, waking time, waking and +30 min cortisol levels, and mean cortisol awakening response divided by *CACNA1C* genotype.

204x147mm (150 x 150 DPI)

Table 3. Correlation table showing partial r, excluded pairwise values for *CACNA1C* methylation levels at CpG sites 1-11 and waking cortisol, cortisol awakening response, and current perceived stress. N=101, controlling for age

Variable	CpG1	CpG2	CpG3	CpG4	CpG5	CpG7	CpG8	CpG9	CpG10	CpG11	MM
Waking cortisol	-.15	-.27**	-.14	-.05	-.10	-.13	-.14	-.22*	-.25*	-.11	-20*
Cortisol awakening response	.01	-.10	.08	.04	-.03	.05	.04	.02	-.03	-.02	-.03
Perceived Stress (PSS-14)	.19	.10	.14	.14	.21*	.18	.15	.13	.14	.08	.12

Notes: MM= mean methylation % *p< .05; **p < .01.

Table 3 Partial correlational analysis showing r values for *CACNA1C* methylation levels at CpG sites 1-11 (without CpG6; n=97-101), overall mean methylation (MM without CpG6) and waking cortisol, cortisol awakening response, and current perceived stress adjusting for age.

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